Temporal variation in cellular proliferation during recornification of mouse tail skin

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Abstract. The influence of the time of injury on subsequent epidermal regeneration is unknown. Epidermal cell proliferation of tail skin in C57BL/6J mice in response to tape stripping was followed for 7 days by radiolabelled thymidine incorporation and autoradiography. The homeostatic labelling index (LI) of the basal epidermis of unmanipulated, unwounded (control) animals was 7.6% and did not vary depending on the time of day. Tape stripping increased the LI of epidermal basal cells 110% above control values 24 h after injury. Labelling indexes of epidermal basal cells in the skin adjacent to the wounded area were 7.0%. Basal cell DNA synthesis stimulated by wounding exhibited a distinct temporal variation at 24 h postinjury, with tail skin wounded at 12.00 h found to be 275% greater than control values and elevated 78% from LIs recorded at any other time point. This temporal spike was due to the time of day at which wounding occurred rather than the time point when the LI was determined. Mice wounded at 12.00 h and terminated 27 h later (15.00 h) had LIs that were 52% greater than wounds created at 09.00 h and examined at 12.00 h the following day. Higher levels of DNA synthesis in tail skin injured at 12.00 h compared to wounding at 09.00 h was detected 12–48 h after injury. Furthermore, DNA synthesis in wounds created at 12.00 h returned to baseline levels 1–2 days earlier than tail skin wounded at 09.00 h. Investigation of other strains of mice detected differences in radiolabelling of epidermal basal cells 24 h after tape stripping at 12.00 h or 09.00 h in CD-1 and BALB/cJ mice, but not in the C3H/HeJ strain. These results indicate: (a) there is no diurnal variation in the LI of mouse tail skin under normal homeostatic conditions (b) tape stripping is a potent stimulator of basal cell turnover in the epidermis (c) the time of wounding determines the magnitude of the increase in the LI of basal cells following injury, and (d) the proliferative response to wounding of the tail is dependent on the strain of mouse.

INTRODUCTION

The epidermis contributes to the protective function of the skin by maintaining a layer of cornified cells, the stratum corneum. Removal of the stratum corneum by repetitive stripping

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with adhesive tape stimulates regeneration of the layer by a vigorous increase in the proliferative activity of the basal cell layer (Pinkhus 1951, Potten & Allen 1975, Peacock 1984). Moreover, maximal mitotic activity occurs between 48 h and 72 h following stripping in both man and mice (Pinkhus 1951, Van Winkle 1968, Potten & Allen 1975, Peacock 1984).

The existence of a circadian rhythm in the homeostatic mitotic activity of mouse epidermis has been reported (Bullough 1948, Grube *et al.* 1970, Hegazy & Fowler 1973, Potten *et al.* 1977, Kietzmann *et al.* 1990). Reasons proposed for the diurnal variation observed, include changes in the duration and/or rate of mitosis, alteration in the number of cells entering DNA synthesis, or the presence and influence of modulators in the cell cycle (Grube *et al.* 1970, Potten *et al.* 1971b, Potten 1975). The timing of peak DNA synthesis and mitotic activity for the epidermis varies between strains of mice (Grube *et al.* 1970, Potten *et al.* 1971b, Potten *et al.* 1977, Kietzmann *et al.* 1990) and body location (Potten *et al.* 1971b). Compared to epidermis of the dorsum or ear, the smallest diurnal variation in the labelling index (LI), was found in mouse tail skin with reversal of the timing of peak synthesis (i.e. higher labelling during day than night) (Potten 1975). Cell proliferation in epithelial tissues has also been demonstrated to be influenced by daily variations of metabolic activity (Garte & Belman 1980, Harris & Mackenzie 1982, Kietzmann *et al.* 1990), responsiveness to carcinogens (Iversen *et al.* 1970, Clausen *et al.* 1984, Iversen & Iversen 1995), and growth factors (Zagon *et al.* 1996, Zagon *et al.* 1997).

The effect of injury on the circadian rhythm of cell replication in the epidermis is uncertain. Peacock (1984) states that wounding abolishes any diurnal variation in mitotic activity of the epidermis. However, injury to the rectal mucosa did not interfere with diurnal variation in mitotic activity (Reeve 1975), and circadian rhythm influenced DNA synthesis and mitotic activity in regenerating rat corneal epithelium (Sandvig *et al.* 1994). Removal of hair and cornified cells of mouse dorsum skin results in an oscillatory pattern of peaks and troughs in DNA synthesis after injury (Hennings & Elgjo 1970, Potten 1971, Potten *et al.* 1971b, Potten *et al.* 1971a, Potten & Allen 1975). Of the aforementioned reports, only one controlled the time of wounding (Hennings & Elgjo 1970), whereas all other studies maintained a set time of day for termination of mice and collection of epidermis for investigation.

The present study was designed to address the question of whether cell proliferation depended on the time of day at which injury occurs. Utilizing a detailed analysis of DNA synthesis in epidermal basal cells with regard to the time of wounding, the results indicate that the magnitude of DNA synthesis in tail wounds does not follow a circadian rhythm, but was dependent on the time of injury. Additionally, the temporal course of recornification was associated with the time of wounding, and the proliferative response to injury was related to the strain of mouse examined.

MATERIALS AND METHODS

Inbred C57BL/6J, C3H/HeJ, BALB/cJ (The Jackson Laboratory, Bar Harbor, ME) and outbred CD-1 (Charles River Laboratories, Wilmington, MA), specific pathogen-free male mice, 7–9 weeks in age, were used in this study. Mice were housed in a cubicle, separated from other rodents, in solid bottom metal cages (three mice per cage) in an environment of 21 (0.5 °C with a relative humidity of 50 (10%. The room had a complete exchange of air 15–18 times/h and a 12:12-h light-dark cycle (light from 07.00 to 19.00 h) with no twilight.

Food (Teklad Rodent Diet #8604, Harland-Teklad, Madison, WI) and chlorinated water were provided *ad libitum*.

The method of wounding was a modification of procedures reported by Stoscheck *et al.* (1992) in which hair was not removed prior to tape stripping. Mice were anaesthetized by intraperitoneal (i.p.) injection of ketamine (Ketaset®, Fort Dodge Laboratories Inc., Fort Dodge, IA; 100 mg/kg) and xylazine (Xyla-Ject®, Phoenix Pharmaceutical Inc., St. Joseph, MO; 7 mg/kg). Cloth tape (Durapore®, 3 M Corp., Minneapolis, MN), 1 cm wide, was wrapped around the tail 1 cm from its base. The tape was removed immediately and this process repeated for a total of 10 times. Wounds were created at 09.00 or 12.00 h in C57BL/6J mice and the animals terminated at 6, 12, 24, 27, 36, 48, 72, 96, 120, 144 or 168 h later. Separate groups of C57BL/6J mice were wounded at 06.00, 13.00, 15.00, 18.00, 21.00 and 24.00 h and terminated 24 h later. CD-1, C3H/HeJ and BALB/cJ mice were injured at 09.00 and 12.00 h, were terminated 24 h later. All experiments with mice were carried out with the approval of the Institutional Animal Care and Use Committee of The Pennsylvania State University College of Medicine.

Thirty minutes prior to killing, mice were injected i.p. with [³H]-thymidine (5 µCi/g body weight, specific activity 20 Ci/mmol.; DuPont-New England Nuclear, Boston, MA). Mice were terminated by cervical dislocation. The entire tail of each mouse was removed and immersed in 10% neutral buffered formalin for 24 h. After fixation, the dorsal skin of the tail was removed by making a longitudinal incision on both sides of the tail and stripping the skin from the underlying fascia. The wound was bisected and 6–8 mm of adjacent unwounded skin was included. Paraffin-embedded tissue was sectioned perpendicular to the surface at 6 µm, coated with NTB-2 emulsion (Eastman Kodak Co., Rochester, NY) and stored in light-tight boxes at 4 °C. After 17–19 days of exposure, the tissues were developed with Kodak D-19 (Eastman Kodak Co., Rochester, NY) and counterstained with Mayer's haematoxylin and eosin. The number of cells (at least 300 cells/wounded or unwounded region/section) with four or more grains (background was one grain/cell) in the basal epithelium was counted from two sections/mouse at each time point. The interfollicular epidermis was evaluated and the hair follicles were avoided. At least three mice were examined at each time point.

Labelling indexes were computed as the number of labelled basal cells divided by the total number of basal cells with nuclei $\times 100$. Data were analysed using analysis of variance (BMDP Statistical Software, Inc., Los Angeles, CA), and subsequent comparisons made using Newman–Keuls tests. Two-sample *t*-tests were used to compare LIs in wounds made at 09.00 or 12.00 h at specific intervals after injury.

RESULTS

Tape stripping of the mouse tail produced a discrete wound, with all hair and 75–80% of the cornified layer removed; underlying layers of the epidermis remained intact (Fig. 1A,B). In order to formulate an assessment of DNA synthesis in C57BL/6J mice following this method of wounding, determination of the structure of the epidermis and the location of cells undergoing DNA synthesis within the area of study was required. In the interfollicular region of unwounded tail skin, the stratum basale comprised a single layer of dense cuboidal-to-columnar shaped cells with their long axis orientated perpendicular to the underlying basal lamina (Fig. 1A). Mitotic figures were observed in the stratum basale. The stratum spinosum was 2–3 cells in depth. The stratum corneum, located at the surface of the epidermis and superior to the stratum spinosum, consisted of many flattened, fused cellular remnants that

no longer possessed a nucleus. A distinct stratum granulosum was not present in the interfollicular epidermis of unwounded tail skin in C57BL/6J mice.

Subtle changes in the cellular architecture of the interfollicular region in the wounded portion of the epidermis were noted after injury (Fig. 1B). Twenty-four hours after injury, the basal cell nuclei changed from a cuboidal/columnar to a spherical shape and were of a

Figure 1. Photomicrographs of longitudinal sections of dorsal tail skin from C57BL/6J mice adjacent to (A) or in the wounded region (B–F). (A and B) 24 h; (C) 48 h; (D) 72 h; (E) 96 h; (F) 168 h after tape stripping. Paraffin-embedded sections of 6 μ m were stained with Mayer's haematoxylin and eosin and photographed. Arrow indicates representative cell labelled with [3 H]-thymidine. In panel A, components of the epidermis, the stratum corneum (sc), stratum spinosum (ss), and stratum basale (sb) are identified. Scale bar (F): 65 μ m.

larger diameter than basal cell nuclei of unwounded skin. Cells labelled with [^3H]-thymidine, as well as occasional mitotic figures, were limited to this singular layer. At 48 h after injury, 30–50% of basal cell nuclei were labelled (see also Fig. 3a,c). Occasionally, cells containing radiolabel were noted in the stratum spinosum. At 72 h after injury, the number of [^3H]-thymidine labelled basal cells was reduced by 30–50% compared to 48 h after injury. Mitotic figures were frequently noted in the stratum basale at 72 h, and appeared to be more common than at any other interval sampled. The basal cells at 72 h postinjury were of a tall columnar configuration. At 48–96 h after injury, parakeratosis and a stratum granulosum were frequently observed in the wounded region (Fig. 1C).

The LI of the epidermal basal cells in tail skin of unanaesthetized C57BL/6J mice (control) was 7.6 (0.4%). Twenty-four hours following injury at 12.00 h, DNA synthesis of basal cells was elevated 4-fold above the control value (Fig. 2). DNA synthesis in epidermal basal cells in skin adjacent to the wounded region was unaffected by injury (7.9 (0.8%)) and was comparable to control levels. Anaesthesia of mice without wounding had no effect on the LI of epidermal basal cells (7.1 (0.6%)).

Experiments were performed to evaluate whether DNA synthesis in tail skin occurs in a circadian rhythm-dependent fashion. Investigations of autoradiographic preparations sampled at 3-h intervals across an 18-h time span revealed that DNA synthesis in tail skin of control animals did not exhibit a daily variation (Fig. 2). Elevations of the LI in response to wounding did display a temporal variation (Fig. 2B). DNA synthesis in epidermal basal cells 24 h after injury at 06.00, 09.00, 15.00, 18.00, 21.00 or 24.00 h was 94–156% greater than control values. However, DNA synthesis by basal cells 24 h after injury at 12.00 h was significantly different from all other intervals sampled and was 275% greater than control. Tail skin adjacent to the wounded region was unaffected by wounding, and the LI was comparable to that of control specimens (Fig. 2C). Determination of whether the time of injury or the time of examination, was related to the magnitude of labelling was investigated. Twenty-seven hours following injuries at 09.00 or 12.00 h there was up to a 46% reduction in LI from wounds examined at the 24 h timepoint. However, wounds made at 12.00 h and examined 27 h later (i.e. 15.00 h) had 52% more radiolabel (15.3 (1.4%)) than wounds created at 09.00 h and examined at 12.00 h the next day (10.0 (1.3%)).

Labelling indexes of wounds made at 12.00 h were higher than those of tail skin wounded at 09.00 h for 12–48 h after injury (Fig. 3A,C). Twelve hours after injury at 12.00 h, the amount of radiolabelled basal cells was 20% higher than control levels and 180% greater than values for wounds made at 09.00 h. In contrast, 12 h following injury at 09.00 h, a 58% decrease in DNA synthesis compared to control values was detected. By 144–168 h after injury, DNA synthesis in wounds produced at 12.00 h was similar to control levels. However, even at 168 h after injury, labelling in wounds created at 09.00 h were 53% higher than control values.

In general, DNA synthesis in the epidermis adjacent to the region of injury was unaffected (Fig. 3B,D). The only significant increases in the LIs of adjacent unwounded skin were detected at 36 and 96 h after injury at 12.00 h.

To examine whether time of wounding was strain dependent, wounds were created at 09.00 and 12.00 h and examined 24 h later in mice of three common inbred strains and one outbred stock (Table 1). No change in LI was detected between wounds created at 09.00 and 12.00 h in C3H/HeJ mice. However, a 37% decrease in LI was noted in wounds of BALB/cJ mice created at 12.00 h in comparison to those made at 09.00 h. Finally, tape stripping at 12.00 h resulted in LIs that were 24% and 73% greater than 09.00 h in CD-1 and C57BL/6J mice, respectively.

Figure 2. Histograms of the labelling indexes of cells (i.e. percentage of labelled cells) in the basal layer of dorsal tail epidermis from C57BL/6J mice. Specimens were evaluated at each time point in unanaesthetized, uninjured control animals (A), or 24 h after repetitive tape stripping for each time point of wounded skin (B) or adjacent, unwounded skin (C) in anaesthetized, injured mice. [^3H]-thymidine was given 30 min prior to termination; at least 1000 cells/time point were counted for control animals and 3000 cells per unwounded and wounded regions for each time point counted in injured mice. Error bars, mean $\pm$ SEM. ** Significantly different from control value at $P < 0.01$ (control, $n = 3$ animals/time point; manipulated mice, $n = 5$ animals/time point). † Significantly different from all other time points, $P < 0.01$.

DISCUSSION

In this study, evidence is provided that the time of wounding of mouse tail skin is a critical determinant of the magnitude of DNA synthesis in basal cells of the epidermis during repair. Thus, 24 h after tape stripping of the tails of C57BL/6J mice, wounds created at 12.00 h had levels of DNA synthesis that were ≈ 2 -fold higher than values detected at any other time of day. The time of wounding also was found to correlate with the temporal return to normal levels of DNA synthesis in the epidermis. For example, epidermis injured at 12.00 h returned to baseline values for DNA synthesis 1–2 days earlier than wounds created at 09.00 h. These

Figure 3. Histograms of the labelling indexes of cells (i.e. percentage of labelled cells) in the basal layer of dorsal tail skin from C57BL/6J mice. Wounds were created at 09.00 h (A—wounded region; B—adjacent unwounded region) or 12.00 h (C—wounded region; D—adjacent unwounded region) and skin sampled at various intervals after wounding. [^3H]-thymidine was given 30 min prior to termination; at least 1800 cells were evaluated at each time point. Error bars, mean $\pm$ SEM. Control (Ctrl) values were determined in unanaesthetized, unmanipulated animals terminated at 09.00 h or 12.00 h ($n=3$ animals/time point). Significantly different from respective control value at $P<0.05^*$ or $P<0.01^{**}$ ($n=3$ animals/time point except 24 h, where $n=5$ animals). $\dagger$ Significantly different from respective control value at $P<0.05^*$ or $P<0.01^{**}$ ($n=3$ animals/time point except 24 h, where $n=5$ animals). $\ddagger$ Significantly different from animals wounded at 09.00 h ($P<0.05$); 2-sample t -test.

Table 1. Dependence of percentage of radiolabelled basal cells in wounds created at 09.00 h or 12.00 h with regard to strain of mouse utilized

Strain	Labelling index (%) ^a	
	09.00 h	12.00 h
C57BL/6J	16.5 ± 1.2 ^b	28.6 ± 1.9**
CD-1	10.8 ± 0.7	13.3 ± 0.7**
BALB/cJ	8.7 ± 0.7	5.5 ± 0.6**
C3H	7.3 ± 0.7	6.6 ± 0.5

^aPercentage of labelled cell nuclei in the basal layer of dorsal tail skin 24 h postinjury of different inbred (C57BL/6J, C3H, BALB/c) or outbred (CD-1) strains of mice. [³H]-thymidine was given 30 min prior to termination; at least 1800 cells were evaluated at each time point for each group.

^bMean ± SEM. Significantly different from animals wounded at 09.00 h, $P < 0.01^{**}$ ($n = 3$ animals per time point except C57BL/6J strain where $n = 5$).

findings are consonant with, and extend, data presented in earlier studies suggesting a correlation between the time of injury and mitotic activity in rat rectal mucosa (Reeve 1975), rabbit epidermis (Blumenfeld 1943), guinea pig tympanic membrane (Reeve 1977) and rat mesothelium (Sapozhnikova 1985). Thus, the temporal aspects of injury to repair processes of the mammalian epithelium appears to be an important influence in the course of wound healing.

The model utilized in this investigation, tape stripping of the mouse tail, provided a reliable, reproducible and highly localized method of evaluating epidermal reepithelialization, and is consistent with the results of Stoscheck *et al.* (1992). The present study focused on the interfollicular basal layer of cells in order to eliminate errors introduced by the variations in DNA synthesis occurring in different layers of the epidermis. These results confirm the structural attributes of the interfollicular epidermis of tail skin in C57BL/6J mice in homeostasis and following injury (Schweizer & Marks 1977, Marks *et al.* 1983). The stratum basale is the proliferative layer of this region and concurs with previous reports (Montagna & Parakkal 1974, Lever & Schaumburg-Lever 1990, Stoscheck *et al.* 1992).

A number of mechanisms may be considered to account for the dependency of the magnitude of DNA synthesis in mouse tail epidermis with respect to the time of injury. First, the repair of damaged epidermis may be influenced by an existing circadian rhythm in DNA synthesis of the basal cells in the homeostatic condition present at the time of wounding. Our investigations demonstrated that diurnal variation in DNA synthesis does not exist in basal cells comprising tail epidermis in homeostasis. This finding is in agreement with the results of Potten (1975) who reported minimal variation in LIs of epidermal cells of tail skin in DBA-2 mice over a 24-h period.

The dependence of the magnitude of DNA synthesis on time of injury identified in this investigation only occurred in cells within the wounded region, and not in epidermal basal cells adjacent to the site of tape stripping. This finding leads us to postulate that the time of wounding sensitized injured cells to diurnal changes in local factors, intracellular events and/or cellular kinetics within the damaged epidermis. RNA synthesis increases rapidly (within 10 h) in wounded tissue and precedes the increase in DNA synthesis (Potten 1971, Oehmichen & Lagodka 1992), presumably accounting for the postwounding suppression of radiolabelled thymidine incorporation (Hennings & Elgjo 1970, Potten 1971). In the present study, postwounding suppression of DNA synthesis was only identified in wounds created at 09.00 h. This would suggest a possible difference in the rate of RNA synthesis in wounds

created at 12.00 h compared to those made at 09.00 h. Another explanation for the high rate of DNA synthesis in epidermis injured at 12.00 h relates to the timing of diurnal variation for epidermal metabolism (Marks & Grimm 1972, Garte & Belman 1980, Harris & Mackenzie 1982). Cyclic nucleotides have antagonistic effects on epidermal proliferation; cAMP being inhibitory and cGMP being stimulatory (Garte & Belman 1980). Cyclic GMP has been shown to peak in mouse epidermis at the midpoint of the animal's light cycle (Garte & Belman 1980). Thus, injury of tail skin at 12.00 h may have coincided with a peak in cGMP-stimulated basal cells.

Changes in the number of cells, or the flux of cells into different subpopulations of the basal layer may account for the difference in DNA synthesis observed between 12.00 h and 09.00 h. Evidence for kinetic heterogeneity of the epidermal basal cell population has been presented elsewhere (Potten 1986, Clausen *et al.* 1986a, Kirkhus & Clausen 1990, Kirkhus & Clausen 1992, Aarnæs *et al.* 1993). Variability in the pool of basal cells with proliferative potential has been proposed as one mechanism for circadian variation in mitotic activity (Brown 1991, Kirkhus & Clausen 1992, Aarnæs *et al.* 1993). Our findings and the work of Potten (1975) are in agreement that circadian variation of DNA synthesis is minimal in mouse tail skin, suggesting that there is little variation in the S-phase of epidermal basal cells in the injured region. However, variation in the duration or number of cells in other phases of the cell cycle (e.g. G₀, G₁) could result in more cells being available to enter DNA synthesis when wounds are created at 12.00 h than at other times of day. A dramatically shortened duration of G₁ has been demonstrated in regenerating epidermal basal cells (Morris & Argyris 1983, Clausen *et al.* 1986b) and a causal relationship between the decreased duration of G₁ and loss of normal growth restriction has been advanced (Clausen *et al.* 1986b). Thus, we propose that the higher level of DNA synthesis in wounds created at 12.00 h compared to other times during the day may result from the stimulation of a larger proportion of cells recruited from G₀/G₁ to enter S-phase. Conversely, when tail skin is tape stripped at times during the day other than 12.00 h, a portion of the basal cell population in G₀/G₁ may be retarded from entering S-phase. Further investigations are necessary to substantiate this hypothesis with respect to the cell cycle and epidermal wound healing.

The present study detected a temporal variation in DNA synthesis as a result of the time of injury that was dependent on the strain of mouse investigated. We could not discern a pattern or relationship of the levels of DNA synthesis following injury to the genetic background of the mouse utilized. For example, different levels of DNA synthesis in wounds examined 24 h after injury at 09.00 h or 12.00 h were detected in genetically heterogeneous (outbred stock) and homogenous (inbred strain) mice, as well as in animals of either albino or nonalbino phenotype. In addition, one albino inbred strain (BALB/cJ) had greater radiolabelled thymidine incorporation 24 h after wounding at 09.00 h compared to injury at 12.00 h, showing that the pattern of DNA synthesis in repair need not follow a similar temporal course. In fact, one nonalbino strain (C3H/HeJ) had no difference in the amount of DNA synthesis depending on the time of injury, indicating that cellular renewal does not have to rely on any temporal factors. Thus, these data would imply that the strain of animal as well as the time of wounding are important factors to consider in designing investigations that explore epidermal regeneration. Therefore, the results of earlier studies related to damage of the epithelium need to be interpreted in light of these new observations.

Given that the level of DNA synthesis attained by healing epidermis is reliant on the time of day that wounding occurred and the strain of mouse utilized, failure to control for these influences may obscure or exaggerate analysis of cellular replication in response to injury. Few reports (e.g. Potten 1971) have identified the time of wounding, much less controlled for

this critical factor. Differing levels of DNA synthesis or mitosis reported for the proliferative response and the degree of oscillatory changes following wounding may be explained in part by considering the time at which the wound was created. This time dependence is also important for future studies with growth modulators because some factors are known to be active at periods of maximal or minimal cell proliferation. Finally, it is of interest to speculate that our findings may indicate a need in the clinical setting to be aware that the timetable of surgery and injury could be related to repair processes.

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